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THE ACTION OF CERTAIN SALTS ON THE COMPLEMENT IN IMMUNE SERUM.*

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IN a recent article[1] Dr. Hektoen has pointed out that certain salts possess the property of suspending the hemolytic action of normal human serum on rabbit's corpuscles and its bacteriolytic action on typhoid bacilli. He has further shown that this suspension is probably due to the action of certain products of the ionization of these salts on the complement in the serum. At his suggestion, I have undertaken to determine whether or not a similar suspension takes place in an artificially immunized serum, and, if so, to inquire somewhat further into the chemistry of the process.

The work forming the basis for this report was done at the University of Chicago during the summer of 1903. Limitations in time have necessarily left it incomplete in many of its phases, but enough has been accomplished to show that there is the possibility of much valuable—and perhaps practical—information coming from a further pursuance of this line of investigation.

Material and apparatus.—The serum used for the study was obtained by immunizing a goat against dog's corpuscles, by weekly intraperitoneal injections of defibrinated dog's blood. One week after the third injection the serum was strongly hemolytic to dog's corpuscles. A record of the injections and hemolytic powers is shown in Table I.

To obtain the serum, blood was drawn from the jugular vein of the goat by means of the apparatus described by Portis on p. 129 of this issue. The blood was allowed to coagulate, and was then put in a refrigerator till the serum separated. This usually took place in about two hours. The serum thus obtained was generally perfectly clear. The few corpuscles that sometimes

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[1] *Trans. of the Chi. Path. Soc.*, 1903, 5, 303-309.

remained in it were removed by centrifugation. The dog's blood was drawn in a somewhat similar apparatus, was defibrinated by beating it with a sterile glass rod, and placed in a refrigerator till used. In drawing blood, of course, every aseptic precaution was taken.

TABLE I.

Record of Injections and Hemolytic Power of Serum.

DATE	INJECTIONS	HEMOLYTIC POWER[2]		REMARKS
		Unwashed Corpuscles	Washed Corpuscles	
1903				
June 30.....	20 c.c. dog's blood	0+%	..	
July 6.......	25 c.c. dog's blood	6	..	
13.......	30 c.c. dog's blood	25	..	
20.......	35 c.c. dog's blood	95	..	Marked agglutination
August 3....	20 c.c. dog's blood	...	55%	
17....	20 c.c. dog's blood	...	55	
24...	....	...	..	Unstable c'mplem'nt[3]
29....	30 c.c. dog's blood	100	60	
September 7.	....	...	57	Compl't again stable

During the earlier experiments considerable difficulty was experienced in handling the dog's corpuscles. These are very delicate, and often broke up spontaneously in physiological saline. To avoid this the following precautions were afterward taken:

1. Blood was taken when possible only from strong, robust dogs, apparently in good health and spirits.

2. Blood was drawn in much larger quantities than needed for the experiments. It was found that larger quantities could be defibrinated with greater ease and apparently with less harm to the corpuscles.

3. The corpuscles were allowed to stand for an hour before being separated from the serum. This was for the purpose of

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[2] Measured according to the scale proposed by WENSTRAND and described by him in the *Trans. of the Chi. Path. Soc.*, 1903, 5, 288-298.

[3] Serum drawn on the afternoon of August 17 gave, the next morning, 55 per cent. hemolysis with washed corpuscles and 20 per cent. hemolysis after it had been standing in the refrigerator for four days. Serum drawn August 24, August 26, and August 29 almost completely lost its hemolytic power for washed corpuscles in twenty-four hours, and completely lost it in two days. The hemolytic power for unwashed corpuscles, however, remained intact, showing that the loss was probably due to a rapid degeneration of the complement, the dog's complement being capable of reactivating the serum. A week later the normal keeping quality of the serum had returned. No reason could be found, either in technic or care of animal, to account for this temporary instability of the complement.

allowing the bactericidal substances in the serum time to act on any possible contaminating organisms.

4. In handling the corpuscles, all unnecessary shaking, stirring, etc., was avoided. It was found that rough treatment readily broke up the corpuscles.

5. The blood was generally used within two hours of its time of withdrawal, never later than three hours.

With these precautions it was found possible to keep dog's corpuscles in physiological saline for several days without more than a slight yellow tint appearing in the supernatant liquid, a tint that is recorded in subsequent experiments as 0+ per cent. hemolysis.

The salt solutions used in the investigation were all of the same concentration, one-eighth gram molecular ($\frac{m}{8}$). They were made from chemically pure salts and water redistilled from glass. Before being used the solutions were sterilized in an autoclave (115°C. for seven minutes), care being taken to make the necessary evaporation as small as possible and uniform for the various solutions. All glassware used in the work was rendered chemically clean by a long treatment with alkali, acid, and distilled water, was thoroughly dried, plugged with absorbent cotton, and sterilized by dry heat (140°C. for one hour).

Method.—To test the effect of a salt solution on the hemolytic power of the serum, varying amounts of the solution were measured out into small test-tubes (3 c.c.), enough physiological saline added to make the volumes up to a uniform amount (1.0 c.c.), and a given quantity of the serum (0.2 c.c.) added to each. The strictest aseptic technic was, of course, employed. The resulting mixtures were thoroughly shaken and allowed to stand for an hour to permit a thorough interaction of the salt and serum. A uniform amount (0.5 c.c.) of a 20 per cent.[4] suspension of washed dog's corpuscles was then added to each mixture and the tubes incubated at 37.5°C. for two hours. They were then placed in a refrigerator, and about fifteen hours later the amount of hemolysis estimated from the depth of color in the clear liquid above the settled corpuscles.

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[4] Five times the original volume of blood.

With the serum used agglutination was so prompt that, if measures had not been taken to avoid it, the corpuscles would have been clumped in a few minutes at the bottom of the tubes, and thus, to a certain extent, removed from the action of the serum. To prevent this, the test-tubes in the earlier experiments were sealed with sterile paraffin and placed on their sides, so that the corpuscles might agglutinate along the length of the tubes, in a form more accessible to chemical action. In the later experiments a rack was used in which the tubes could be placed in a nearly horizontal position, without sealing. After incubation the tubes were placed in an upright position.

In order to estimate the amount of hemolysis, a color scale was prepared in each experiment by laking the corpuscles used in that experiment in distilled water, in such proportions that 100 per cent. on the scale meant a complete solution of all the corpuscles in each test-tube.

Effects of salts on corpuscles.—According to the above method, the corpuscles were submitted, not only to the action of the modified serum, but to the action of the salt solutions as well. It was necessary, therefore, to know the hemolytic powers of the salt

TABLE II.

Effect of Salt Solutions on Dog's Corpuscles.

(Recorded amounts of salt solution made up to 1.2 c.c. with physiological saline; 0.5 c.c. of a 20 per cent. suspension of washed dog's corpuscles added; incubated for two hours, refrigerator fifteen hours. Record shows percentage of hemolysis.)

SOLUTION	$CaCl_2$	$BaCl_2$	$Na_2C_2O_4$	Na_2SO_4	$MgCl_2$	$Na_2C_2H_4O_6$	NaI	$NaNO_3$	KCl	NH_4Cl	Na_2CO_3	Na_3PO_4
	%	%	%	%	%	%	%	%	%	%	%	%
0.00 c.c. $\frac{m}{8}$ solution.	0+	0+	0+	0+	0+	0+	0+	0+	0+	0+	0+	0+
0.05 c.c. “	7	0+	0+	5−	0+	0+	0+	0+	5−	0+	100	100
0.1 c.c. “	15	0+	0+	0+	5−	0+	0+	0+	5	5−	100	100
0.15 c.c. “	20	0+	0+	0+	5−	0+	0+	0+	5	5−	100	100
0.2 c.c. “	25	0+	0+	0+	5	0+	0+	0+	5+	5−	100	100
0.3 c.c. “	30	0+	0+	5−	7	0+	0+	0+	5+	5−	100	100
0.4 c.c. “	30+	0+	0+	0+	7	0+	0+	0+	7	5	100	100
0.5 c.c. “	35	0+	0+	0+	7	0+	0+	0+	7	5	100	100
0.6 c.c. “	35	0+	0+	0+	7	0+	0+	0+	10−	5+	100	100
0.7 c.c. “	40−	0+	0+	0+	7	5−	0+	0+	10	20	100	100
0.8 c.c. “	40	0+	0+	5−	7	0+	0+	0+	10	20	100	100
0.9 c.c. “	40+	0+	5−	0+	7+	5−	0+	5−	10	80	100	100
1.0 c.c. “	45	0+	5−	0+	10−	5−	5−	5	10+	100	100	100

solutions alone, before attempting to judge their effects on the hemolytic powers of the serum. To determine this, corpuscles were exposed to the action of the salt solutions, the tubes containing them incubated for two hours, placed in a refrigerator, and the percentage of hemolysis estimated colorometrically fifteen hours later, exactly as in the subsequent serum experiments. The results of a number of such experiments are shown in Table II. From this it is seen that, under the conditions of the experiment, $BaCl_2$, $Na_2C_2O_4$, Na_2SO_4, $Na_2C_2H_4O_6$, NaI, and $NaNO_3$ are practically without hemolytic effect on dog's corpuscles; that KCl and $MgCl_2$ produce slight hemolysis; and that $CaCl_2$, NH_4Cl, Na_2CO_3, and Na_3PO_4, have strong hemolytic powers.

Effects of salts on serum.—Experiments were now undertaken to determine the effects of these solutions on the hemolytic powers of the serum. The salt solutions and serum were mixed in varying proportions and the mixture allowed to act on corpuscles in the manner described above. The results are recorded in Table III. From this table it is seen that $CaCl_2$, $BaCl_2$, and $Na_2C_2O_4$ possess the property of rapidly destroying or inhibiting the

TABLE III.

Effect of Salt Solutions on the Hemolytic Action of Immune Serum.

(Recorded amounts of salt solution made up to 1.0 c.c. with physiological saline, 0.2 c.c. of hemolytic serum added, and the mixture allowed to act on 0.5 c.c. of a 20 per cent. suspension of washed dog's corpuscles; incubated for two hours, refrigerator fifteen hours. Record shows percentage of hemolysis.)

SOLUTION	$CaCl_2$	$BaCl_2$	$Na_2C_2O_4$	Na_2SO_4	$MgCl_2$	$Na_2C_2H_4O_6$	NaI	$NaNO_3$	KCl	NH_4Cl	Na_2CO_3	Na_3PO_4
	%	%	%	%	%	%	%	%	%	%	%	%
0.00 c.c. solution..	45	50	55	50	50	45	50	50−	45	45	45	50
0.05 c.c. “ ...	40+	40	50	50	45	30+	50	50	50−	45+	100	100
0.1 c.c. “ ...	25	30+	45	50+	40	35	45	50	45	50	100	100
0.15 c.c. “ ...	20+	25	35	40	35	30	45	50+	45+	50	100	100
0.2 c.c. “ ...	20	20−	20	25	30	20+	55−	50	50	50	100	100
0.3 c.c. “ ...	7	5	5−	20	25	25	50+	50	50	50+	100	100
0.4 c.c. “ ...	5−	0+	0+	15	20+	20−	45+	50+	55	60	100	100
0.5 c.c. “ ...	0+	0+	0+	10−	15	20	45	50	60−	60+	100	100
0.6 c.c. “ ...	0+	0+	0+	5+	10+	10+	45	45	60−	80	100	100
0.7 c.c. “ ...	0	0+	0+	5	10	10+	45	45	60	100−	100	100
0.8 c.c. “ ...	0	0+	0+	5	10	10+	45	45	60	100	100	100
0.9 c.c. “ ...	0	0+	0+	5−	10−	10−	47	45	65	100	100	100
1.0 c.c. “ ...	0	0+	0+	0+	5+	7	50	45	65+	100	100	100

hemolytic powers of the serum—a property that is possessed also by Na_2SO_4, $MgCl_2$, and $Na_2C_2H_4O_6$, though to a less degree. As to the action of Na_2CO_3 and Na_3PO_4 no conclusion can be drawn, as these salts in themselves are such powerful hemolytic agents. KCl and NH_4Cl are probably without effect on the hemolytic power, as the percentage of hemolysis obtained with each mixture is, within the limits of error in such experiments, approximately equal to that of the serum itself plus that of the salt solution. Experiments with Nal and $NaNO_3$ were contradictory, one series showing a slight suspension in hemolytic power, another a slight increase. The recorded results are the averages of two such contradictory series.

Having thus determined that certain salts have the power of suspending the hemolytic action of the serum, the inquiry was next directed to the manner in which they bring about this result. It is evident that there are four ways in which they might do this: (*a*) they might alter the blood corpuscle, physically or chemically, in such a way as to render it insusceptible to the hemolytic action of the serum; (*b*) they might destroy the hemolytic amboceptor, or alter it chemically in such a way as to prevent its union with the corpuscle or with the complement, or otherwise destroy its efficiency in hemolytic action; (*c*) they might destroy the hemolytic complement, or alter it chemically in such a way as to prevent its union with the amboceptor or otherwise destroy its efficiency; and (*d*) it is even conceivable that they might prevent hemolysis by some purely physical action, unassociated with chemical changes in either complement or amboceptor. Experiments were undertaken to test each of these possibilities.

a) Do the salts render the blood corpuscles insusceptible to the hemolytic action of the serum? To test this, washed corpuscles were exposed to the action of the various salt solutions for a couple of hours, were then separated from the solutions by centrifugation, suspended in physiological saline, and submitted to the action of the serum. The results are shown in Table IV. From this it is seen that corpuscles treated with the various salts gave practically the same amount of hemolysis as untreated corpuscles. Unless it is conceivable that the process of centrifugation and subsequent suspension in physiological saline might undo the action of these salts—a conception that further experiments proves to be without foundation—it follows from this that the prevention of hemolysis by these solutions is not due to alterations produced by them in the blood corpuscles.

TABLE IV.

The Effect of Salt Solutions on the Hemolytic Susceptibility of Dog's Corpuscles.

(0.5 c.c. of a 20 per cent. suspension of dog's corpuscles[5] exposed to the action ef recorded amounts of salt solution for two hours; corpuscles then separated by centrifugation and submitted to the action of 0.2 c.c. of hemolytic serum. Record shows percentage of hemolysis.)

SALT SOLUTION	SUBSEQUENT HEMOLYSIS		
	Reading 1	Reading 2	Average
0.5 c.c. $\frac{m}{8}$ $CaCl_2$+0.5 c.c. physiol. saline.	55%	50%	52.5%
0.5 c.c. $\frac{m}{8}$ $BaCl_2$+0.5 c.c. physiol. saline.	55−	55−	55−
0.5 c.c. $\frac{m}{8}$ $Na_2C_2O_4$+0.5 c.c. phy. saline.	50	55	52.5
1.0 c.c. $\frac{m}{8}$ Na_2SO_4	55	50	52.5
1.0 c.c. $\frac{m}{8}$ $MgCl_2$	55	60+	58
1.0 c.c. $\frac{m}{8}$ $Na_2C_2H_4O_6$................	50	50	50
Untreated corpuscles................	50−	55	52

b) Do the solutions destroy the hemolytic amboceptor, or alter it chemically in such a way as to prevent its union with the corpuscle or with the complement, or otherwise destroy its efficiency in hemolytic action? To answer this, hemolytic serum was mixed with the salt solutions in proportions that had been found to suspend hemolysis, and the mixtures allowed to stand, at room temperature, for one and one-half hours. These mixtures were then cooled to 0° C., and at this temperature allowed to act on washed corpuscles.

Normally, at this temperature, the amboceptor unites with the corpuscles, but the complement, possessing a less affinity, does not unite with the amboceptor. As a result no hemolysis takes place, but the corpuscles become sensitized (corpuscle + amboceptor), so that a subsequent exposure of them to pure complement, which in itself possesses no hemolytic power, leads to their solution.

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[5] The experiments, of course, were done on a much larger scale than this, the table recording simply the amounts that entered into each test-tube.

At the end of forty minutes, the corpuscles so exposed were separated from the serum-salt mixtures by centrifugation and washed repeatedly in physiological saline, care being taken to keep the temperature as near 0° C. as possible during the process. The treated corpuscles were then submitted to the action of pure complement (normal goat's serum). The results are recorded in Table V. In all cases practically the same amount of hemolysis took place as in the control experiment with serum not treated with a salt solution. The various salt-serum mixtures are, therefore, capable of sensitizing corpuscles to the same degree as untreated serum. This not only confirms our conclusion of the last experiment that the solutions produce no changes in the corpuscle

TABLE V.

The Effect of Salt Solutions on the Hemolytic Amboceptor.

(0.2 c.c. hemolytic serum added to recorded amounts of salt solution, mixtures allowed to stand one and one-half hours at room temperature, then used to sensitize 0.5 c.c, of a 20 per cent. corpuscle suspension. Corpuscles separated by centrifugation and submitted to the action of pure complement [non-immune goat's serum]. Record shows percentage of hemolysis.)

SALT SOLUTION	SUBSEQUENT HEMOLYSIS BY PURE COMPLEMENT		
	Reading 1	Reading 2	Average
0.5 c.c. $\frac{m}{8}$ $CaCl_2$+0.5 c.c. physiol. saline.	45%	50%	47.5%
0.5 c.c. $\frac{m}{8}$ $BaCl_2$+0.5 c.c. physiol. saline.	40	55	47.5
0.5 c.c. $\frac{m}{8}$ $Na_2C_2O_4$+0.5 c.c. phy. saline.	50	50	50
1.0 c.c. $\frac{m}{8}$ Na_2SO_4	50	45	47.5
1.0 c.c. $\frac{m}{8}$ $MgCl_2$.....................	50	55	52.5
1.0 c.c. $\frac{m}{8}$ $Na_2C_2H_4O_6$................	55	50	52.5
Untreated serum....................	50	50	50

(Pure complement gave with untreated corpuscles 5 + per cent. hemolysis: Sensitized corpuscles not treated with complement gave in each case approximately 5 per cent. spontaneous laking.)

preventing the union of the amboceptor, but also proves that they do not alter the amboceptor in such a way as to prevent its union with the corpuscle. Unless the subsequent suspension of the sensitized corpuscle in physiological saline undoes the action of these salts on the amboceptor attached to the corpuscle, it also follows that the solutions do not alter the amboceptor in such a way as to prevent its union with the complement, or its fulfilling its other functions in hemolytic action.

TABLE VI.

Effect of Salt Solutions on the Hemolytic Complement.

(0.2 c.c. pure complement [normal goat's serum] added to recorded amounts of salt solution; mixtures allowed to stand one and one-half hours at room temperature, and then allowed to act on 0.5 c.c. of a 20 per cent. suspension of sensitized corpuscles. Record shows percentage of hemolysis.)

SALT SOLUTION	SUBSEQUENT ACTION ON SENSITIZED CORPUSCLES		
	Reading 1	Reading 2	Average
0.5 c.c. $\frac{m}{8}$ $CaCl_2$+0.5 c.c. physiol. saline.	5+%	10%	8%
0.5 c.c. $\frac{m}{8}$ $BaCl_2$+0.5 c.c. physiol. saline.	10−	5+	7.5
0.5 c.c. $\frac{m}{8}$ $Na_2C_2O_4$+0.5 c.c. phy. saline.	5+	10−	7.5
1.0 c.c. $\frac{m}{8}$ Na_2SO_4	15−	10	12
1.0 c.c. $\frac{m}{8}$ $MgCl_2$	10−	10+	10
1.0 c.c. $\frac{m}{8}$ $Na_2C_2H_4O_6$	10+	10−	10
Untreated complement	85+	90	88

(Sensitized corpuscles not treated with complement gave 7 per cent. spontaneous laking.)

c) The evidence, therefore, indirectly points either to the complement as being the seat of chemical action, or to the action being a purely physical one. To obtain direct evidence on this point, pure complement was mixed with the salt solutions, and this treated complement afterwards allowed to act on sensitized corpuscles, a control being made with untreated complement. The results are recorded in Table VI. From this it is seen that the sensitized corpuscles gave with the untreated complement 88 per cent. hemolysis, but that with the treated complement there was in each case but a trifle more hemolysis than the spontaneous laking of the sensitized corpuscles (7 per cent.). The experiment furnishes direct evidence that the action of the salts is on the complement, though here again the possibilities of the action being a physical one, or of its being on the amboceptor attached to the sensitized corpuscle, are not ruled out.

In order to rule out definitely the amboceptor as the point of attack, it was attempted to vary the amount of complement in a serum without changing the amount of amboceptor, and to determine for this variable serum the minimum amount of salt necessary to suspend hemolysis.[6] If the amboceptor is the point of attack, the amount of salt should remain constant, no matter how the complement varies. If, on the other hand, the complement is the point of atttack, the amount should vary with variations in the complement.

TABLE VII.

Relation between Amount of Complement and Amount of $CaCl_2$ Necessary to Suspend Hemolysis.

(0.2 c.c. of heated hemolytic serum [pure amboceptor] + recorded amounts of non-immune serum [pure complement] added to recorded amounts of $CaCl_2$ and the volumes made up to 1.5 c.c. by the addition of physiological saline. This mixture allowed to act on 0.5 c.c. of a 20 per cent. suspension of dog's corpuscles. Record shows percentage of hemolysis.)

AMOUNT OF COMPLEMENT	0.00 c.c. *m*/8 $CaCl_2$	0.05 c.c. *m*/8 $CaCl_2$	0.1 c.c. *m*/8 $CaCl_2$	0.15 c.c. *m*/8 $CaCl_2$	0.2 c.c. *m*/8 $CaCl_2$	0.3 c.c. *m*/8 $CaCl_2$	0.4 c.c. *m*/8 $CaCl_2$	0.5 c.c. *m*/8 $CaCl_2$	0.6 c.c. *m*/8 $CaCl_2$	0.7 c.c. *m*/8 $CaCl_2$	0.8 c.c. *m*/8 $CaCl_2$	0.9 c.c. *m*/8 $CaCl_2$	1.0 c.c. *m*/8 $CaCl_2$
0.1 c.c. non-immune serum (pure complement)..........	30	15	10	0+	0	0	0	0	0	0	0	0	0
0.2 c.c. "	50	40	35	20	15	0+	0	0	0	0	0	0	0
0.3 c.c. "	70	60−	50	40	30	10+	5	0+	0	0	0	0	0

Sera with different complement content but constant amboceptor were made by adding to heated hemolytic serum (pure amboceptor) varying quantities of normal goat's serum (pure complement). The results are recorded in Table VII. From this it is seen that with one unit of complement 0.15 c.c. $\frac{m}{8}$ $CaCl_2$

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[6] A second series with constant complement but varying amboceptor was planned, but the time was too short to carry this out.

was required to suspend hemolysis, that with two units of complement 0.3 c.c. $\frac{m}{8}$ $CaCl_2$ was required, and that with three units 0.5 c.c. $\frac{m}{8}$ $CaCl_2$. The ratio of the amounts of complement, 1 : 2 : 3, is approximately equal to that of the $CaCl_2$, 0.15 : 0.3 : 0.5 (=1 : 2 : $3\frac{1}{3}$), and differs from it not more than the errors of observation warrant. It is evident, therefore, that the amboceptor cannot be the point of attack, and that the salts either act chemically on the complement or produce their results by purely physical changes.

TABLE VIII.

Relation between Age of the Serum and Amount of $BaCl_2$ Necessary to Suspend Hemolysis.

(0.2 c.c. of serum was exposed on successive days to the action of recorded amounts of $BaCl_2$ and the mixtures allowed to act on 0.5 c.c. of a 20 per cent. corpuscle suspension. Record shows percentage of hemolysis.)

AGE OF SERUM	0.00 c.c. *m*/8 $BaCl_2$	0.05 c.c. *m*/8 $BaCl_2$	0.1 c.c. *m*/8 $BaCl_2$	0.15 c.c. *m*/8 $BaCl_2$	0.2 c.c. *m*/8 $BaCl_2$	0.3 c.c. *m*/8 $BaCl_2$	0.4 c.c. *m*/8 $BaCl_2$	0.5 c.c. *m*/8 $BaCl_2$	0.6 c.c. *m*/8 $BaCl_2$	0.7 c.c. *m*/8 $BaCl_2$	0.8 c.c. *m*/8 $BaCl_2$	0.9 c.c. *m*/8 $BaCl_2$	1.0 c.c. *m*/8 $BaCl_2$
Serum 1 hour old (Aug. 17)............	100+	100	90+	70	60	40	35	25	0+	0+	0	0	0
Serum 1 day old......	60	60	50	40+	35	7	5−	0+	0	0	0	0	0
Serum 3 days old.....	40	25	15+	10	5−	0+	0	0	0	0	0	0	0
Serum 7 days old.....	20	10	5	0+	0+	0	0	0	0	0	0	0	0

It might be objected to the above experiment, however, that the complement was not the only variable substance in the serum, that, with the extra amounts of normal serum, extra amounts of serum-albumin, serum-globulin, and the like were added. To avoid this criticism, a second series was run in which the volume of the serum mixtures was made up to a uniform amount by the addition of heated, normal goat's serum (containing neither complement nor amboceptor, but containing serum-albumin, serum-globulin, etc.). The results of such an experiment correspond roughly with those recorded above, though here less difference was noted between the amounts of $CaCl_2$ needed with the different amounts of complement. A more convincing experiment, however, was made by testing the same serum on successive days, as the complement degenerated, the amboceptor, presumably, remaining constant during the short time over which the experiments extended. The result of such an experiment is recorded in Table VIII. Here again the same general law holds good that, with variation in the amount of complement, there is a parallel variation in the amount of salt necessary to suspend hemolysis.

We have, therefore, definitely proved that the salt solutions suspend hemolysis either by chemical changes produced by them in the complement, toward which the above experiments strongly point, or by possible physical changes in the liquid. Experiments were next undertaken to determine the fate of the complement in this suspension. Is the complement broken up by the salts, or is the suspension accomplished without its destruction? To determine which of these is true, the inhibiting salt was precipitated from non-hemolytic, serum-salt mixtures, and the hemolytic powers of the remaining liquids tested. The results are recorded in Table IX. In all cases the hemolytic power was restored by such precipitation, and, with two exceptions, practically to the original amount possessed by the serum.

To test whether this return of hemolytic power was due to a liberation of the complement or to possible products of the interaction of the salts used, the liquids were now heated to 56° C. for forty minutes and the hemolytic power again tested. Except in the experiments in which $MgCl_2$ entered, the hemolytic power was destroyed by such heating, showing that, with these two exceptions,[7] the restored power was due to the presence of a thermo-labile substance, undoubtedly the original complement. The experiments, therefore, prove that the suspension of hemolysis is accomplished without destruction of the complement.

d) Evidence ruling out the possibility of the action being a physical one is obtained by comparing the results recorded in

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[7] An examination of the $MgCl_2$ solution showed that, on precipitating the Mg ion with $Na_2C_2H_4O_6$, there was left behind in the liquid a strong thermo-stable, hemolytic agent, the exact nature of which was not determined, but which probably came from a contamination in the $MgCl_2$ solution. It has been suggested that in the process of autoclaving small quantities of HCl might have been driven off from this solution, leaving behind $Mg(OH)_2$, minute traces of which are soluble. On precipitating this with a Na salt, NaOH, a powerful hemolytic agent, would be formed. Time was too limited to test the truth of this suggestion.

TABLE IX.

Effect of Precipitating the Inhibiting Salt.

SERUM	PERCENTAGE OF HEMOLYSIS				SALT SOLUTION	HEMOLYSIS AFTER ADDING SALT SOLUTION				PRECIPITANT[8]	HEMOLYSIS AFTER PRECIPITATION				SAME AFTER HEATING		
	Reading 1	Reading 2	Reading 3	Average		Reading 1	Reading 2	Reading 3	Average		Reading 1	Reading 2	Reading 3	Average	Reading 1	Reading 2	Average
0.2c.c. serum	70	60	65	65%	0.3c.c. $\frac{m}{8}$ $CaCl_2$	7	7	5+	6.3%	0.3c.c. $\frac{m}{8}$ $Na_2C_2O_4$	60%	70%	60%	63.3%	0%	0%	0%
										0.3c.c. $\frac{m}{8}$ $Na_2C_2H_4O_6$	65	65	65	65	0	0	0
					0.3c.c. $\frac{m}{8}$ $BaCl_2$	7	7	7	7	0.3c.c. $\frac{m}{8}$ $Na_2C_2O_4$	65	70	60	65	0	0	0
										0.3c.c. $\frac{m}{8}$ $Na_2C_2H_4O_6$	65	70	65	66.7	0	0	0
										0.3c.c. $\frac{m}{8}$ Na_2SO_4	60	55+	65	60	0	0	0
					0.3c.c. $\frac{m}{8}$ $Na_2C_2O_4$	10	15	10	11.7	0.3c.c. $\frac{m}{8}$ $CaCl_2$	50	50	55	51.7	0	0	0
										0 3c.c. $\frac{m}{8}$ $BaCl_2$	60	70	70	66.7	0	0	0
										0.3c.c. $\frac{m}{8}$ $MgCl_2$	70	75	70	71.7	30	40	35
0.1c.c. serum	30+	30	40	33.3	0.45c.c. $\frac{m}{8}$ $Na_2C_2H_4O_6$	5+	5	5+	5+	0.45c.c. $\frac{m}{8}$ $CaCl_2$	25+	30	30	28.3	0	0	0
										0.45c.c. $\frac{m}{8}$ $BaCl_2$	30	35	30	31.7	0	0	0
					0.45c.c. $\frac{m}{8}$ Na_2SO_4	7	7	7	7	0.45c.c. $\frac{m}{8}$ $BaCl_2$	30	35	30	31.7	0	0	0
					0.45c.c. $\frac{m}{8}$ $MgCl_2$	20	20−	20+	20	0.45c.c. $\frac{m}{8}$ $Na_2C_2O_4$	70	80	70	73.3	40	45	42.5

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[8] With several of the solutions it is necessary to start the process of precipitation by scratching the inside of the test tube with a sterile glass rod.

Tables II and III. From these tables it is seen that $CaCl_2$ possesses strong hemolytic powers, but that, mixed with serum, it not only prevents the hemolytic action of the serum, but loses its own power of hemolysis. It is inconceivable that this could be brought about as long as the $CaCl_2$ remained free in the solution. It is probable, therefore, that the $CaCl_2$, or its active ion, is in some way removed by the serum from an active rôle in the liquid. This would be accomplished if it were bound chemically to the complement.

Further evidence that such a chemical union takes place is furnished by the fact that on heating a non-hemolytic $CaCl_2$ serum mixture, the complement is not only destroyed, as can be proved by the precipitation method described above, but that at the same time, the hemolytic power of the $CaCl_2$ is, in large part, restored. The evidence, therefore, is very strong that the suspending action of the salts is not due to physical changes, but to chemical alterations in the complement.

Since the complement is not broken up by the salts, this action is probably the formation of simple salt or ion compounds with the complement, which compounds are either incapable of union with the amboceptor or hemolytically inactive after such a union.

The salts investigated are all electrolytes, and in the dilutions used are largely ionized. There is reason to believe, therefore, that their action is due to the ions $\overset{++}{Ca}$, $\overset{++}{Ba}$, $\overset{++}{Mg}$, $\overset{--}{SO_4}$, $\overset{-}{C_2}\overset{-}{O_4}$, $\overset{-}{C_2H_4}\overset{-}{O_6}$, their solutions contain, rather than to the salt molecules as a whole. It would be very difficult, however, to prove beyond a question of doubt that such is the case.

CONCLUSIONS.

1. Certain salts, among which are $CaCl_2$, $BaCl_2$, $MgCl_2$, Na_2SO_4, $Na_2C_2O_4$, and $Na_2C_2H_4O_6$, possess the property of inhibiting the hemolytic action of an artificially immunized goat's serum on dog's corpuscles.

2. These salts produce no changes in the corpuscles or in the amboceptor that interfere with hemolytic action.

3. The amount of salt necessary to bring about the inhibition varies with the amount of hemolytic complement in the serum.

4. Precipitation of the inhibiting salts from non-hemolytic, serum-salt mixtures causes a complete return of hemolytic power, which power is destroyed by heating the resulting liquid to 56° C. for forty minutes.

5. There is reason, therefore, to believe that the inhibition is due to the formation of simple salt- or ion-complement compounds, which are either incapable of uniting with the amboceptor or hemolytically inactive after such union.